Applicants also direct the Office to the attached declaration of Dr. Sonya Leyrer where she describes the construction of MVA recombinant virus that contain insertions of more than one serotype into MVA and that such was stable. Reconsideration and withdrawal of the objection to the specification is requested.

Change of Firm Name

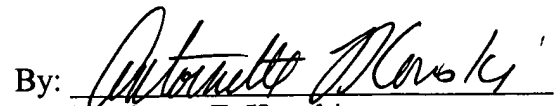
Effective July 1, 2002, the firms of McCutchen, Doyle, Brown & Enersen, LLP and Bingham Dana LLP merged to become Bingham McCutchen LLP.

II. CONCLUSION

If a telephone interview would advance prosecution of the subject application, the Examiner is invited to telephone the undersigned at the number provided below. In the unlikely event that the transmittal letter is separated from this document and/or the Patent Office determines that an extension and/or other relief is required, Applicants petition for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 50-2518**, referencing billing number 20239-0703.

April 3, 2003

Respectfully submitted,

By: 

Antoinette F. Konski
Registration No.: 34,202

Bingham McCutchen, LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
Telephone: (650) 849-4950
Telefax: (650) 849-4800

Atty. Docket No. 20239-0703

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)):

Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Commissioner for Patents, Washington D.C. 20231.

Dated: April 3, 2003 Name of Person Certifying: Peggy Nichols
Printed Name: Peggy Nichols

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: CARDOSA, Mary Jane et al.

Filing Date: March 23, 1999 Examiner: M. Mosher

Serial No.: 09/147,919 Group Art Unit: 1648

Title: **RECOMBINANT MVA VIRUS EXPRESSING DENGUE VIRUS ANTIGENS, AND THE USE THEREOF IN VACCINES**

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF SONJA LEYRER UNDER 37 C.F.R. § 1.132

I, Sonja LEYRER, citizen of Germany, hereby declare that:

1. I have a Diploma degree in Biology (Dipl. Biol.) and a Ph.D. in Virology, both from the University of Munich. From 1993 on I have conducted research in the field of virology. During my Ph.D thesis from 1994 to 1998, I used recombinant Vaccinia viruses as helper viruses in a system for the generation of recombinant negative strand RNA viruses. Since 1999 I have been employed by Bavarian Nordic GmbH as scientist in the field of Poxvirology, i.e. in the Pox Vectored Vaccine Development. My current position (since January 2001) is Manager of the Department for Pox Vectored Vaccine Development. I am responsible for the generation of recombinant Modified Vaccinia Virus Ankara (MVA). Under my guidance, 43 recombinant MVA have been designed and developed for vaccine development and basic research. It is also within my responsibility to develop all assays necessary for the quality control in recombinant MVA production. My curriculum vitae is set forth in Appendix A to this Declaration. In view of the above, I believe that I am one of skill in the art in the subject matter of the above-identified application.

2. I have read the subject application papers and the Final Office Action issued November 30, 2001.

3. All experiments disclosed in this Declaration were conducted by me or by Sonja Scherzinger while acting under my constant guidance and supervision. Sonja Scherzinger has been employed since 1999 as a technical assistant in the department for Pox Vectored Vaccine Development by Bavarian Nordic GmbH.

4. The experiments as disclosed in the present Declaration were made to analyze (i) whether recombinant Modified Vaccinia Virus Ankara (MVA) can be generated that comprises homologous genes from two or more Denguevirus serotypes, i.e. sequences encoding the PrM of two or more different Denguevirus serotypes and (ii) whether the recombinant viruses comprising two or more homologous sequences are stable.

5. Using the subject specification as a guide as to how to make and use the MVA, the materials were prepared, as set forth in paragraphs 6 through 14, infra.

6. Primary CEF cells: SPF-eggs were received fertilized from SPAFAS and stored not longer than 12 days at 4°C. The eggs were incubated for 10 - 12 days at 37.8° C ± 0.8° C and 60 % ± 10% relative humidity. Eggs were placed in a dedicated egg carton and treated extensively with Bacillol. The shell was removed carefully and the chorioallantoic membrane was put aside. The embryos were decapitated and the remains transferred into a petri-dish filled with PBS. The feet were removed and the trunks were transferred to a petri dish containing 10-20 ml PBS. 11 trunks at maximum were given in a 20 ml plastic syringe and squeezed in an Erlenmeyer flask. 5 ml pre-warmed (37°C) Trypsin/EDTA-solution were added per trunk. The solution was stirred for 15 min with medium speed at RT using a magnetic stirrer. The trypsinized cells were poured from Erlenmeyer flask through 1 layer of mesh into two different beakers. All cells were transferred to one 225 ml centrifuge tube and spun down at 20°C, 470 x g, 7 min in a centrifuge. Supernatant was discarded and pellets were resuspended in 1 ml fresh pre-warmed (37°C) VP-SFM per trunk by pipetting thoroughly. Pre-warmed (37°C) medium to a total volume of 150 ml was added. The centrifugation step was repeated and the

cells were resuspended thoroughly. The required amount of cells was seeded in the desired cell culture vessels containing the appropriate medium and incubated at 37°C.

7. 1st passage CEF: In order to obtain 1st passage CEF cells medium from primary CEF was removed and cells were washed once with PBS. Trypsin/EDTA was added and equally distributed over the cells. After an incubation period at 37°C for up to 10 min cells were detached. Relevant medium to a total volume of 10 ml was added cells were resuspended with pipette and transferred into a centrifuge tube. Spin cells down at room temperature, 5 min, 250 g. The supernatant was discarded and the cells were re-suspend in an appropriate volume of medium.

8. The MVA strain: MVA-BN virus was obtained from laboratory reference stocks with defined titer and is disclosed in the International Patent Application WO 02/42480. Other than being more attenuated than the strain disclosed in the subject specification, the strains are identical.

9. Plasmids: For expression of foreign genes by recombinant MVA (recMVA) these genes were inserted into the deletion sites of the viral genome of MVA by homologous recombination using the methods disclosed in the subject specification. To insert Denguevirus PrM genes into the genome of MVA-BN, the PrM genes of the four serotypes of Dengue were cloned into different integration vectors (recombination vector) in such a way that the PrM genes were flanked by MVA sequences homologous to the intended insertion site in the MVA genome. The recombinant vector may also contain a selection and/or reporter cassette for detection of infected and transfected cells. As used in this Declaration, the terms PrM1-4 are used for the PrM genes/proteins of Denguevirus subtypes 1-4. A schematic representation of the recombination plasmids pBN49 for the insertion of PrM1 in Deletion 2, pBN39 for the insertion of PrM2 in Deletion 1, pBN50 for the insertion of PrM3 in the intergenic region (IGR) I4L-I5L and pBN40 for the insertion of PrM4 in Deletion 4 is shown in Figure 1 as attached to this Declaration as Appendix B.

10. Reagents for Transfection, Cell culture medium and substrates: Transfection was conducted as set forth in the subject specification. For serum free cell culture processes

VP-SFM medium (Gibco BRL, Germany, Karlsruhe) containing L-Glutamin (4mmol/l; Gibco BRL, Germany, Hilden) was used. The selection media for Neomycin expression was Dulbecco's Modified Eagles Medium (Gibco BRL, Germany, Hilden) containing 300 µg G418 (Gibco BRL, Germany, Hilden) per ml. For Ecogpt expression the selection media was Dulbecco's Modified Eagles Medium (Gibco BRL,Germany, Hilden) containing 25 µg/ml Mycophenolacid (Sigma, Germany, Taufkirchen), 250 µg/ml Xanthin (Sigma, Germany, Taufkirchen) and 15 µg/ml Hypoxanthin (Sigma, Germany, Taufkirchen). Optionally the media comprised 2% low melting agarose mixed with the appropriate selection medium (1:1). PBS was purchased from Gibco BRL, Germany, Hilden.

11. Reagents for PCR screening, DNA extraction and sequencing: The Taq Polymerase kit as well as the dNTP set (PCR grade) were obtained from Roche, Germany, Mannheim. Oligonucleotides were ordered from MWG, Germany, Ebersberg. For the screening of MVA-mBN12, the following oligonucleotides were used: oBN93 (cgcggatcca tgctgaacat cttgaacagg agacgcaga) and oBN477 (catgataaga gattgtatcag) for amplification of PrM2 in the deletion site 1 of the MVA genome (Meyer H., Sutter G. and Mayr A. J Gen Virol. 1991 May; 72 (Pt 5) :1031-8.); oBN210 (atcccattcc tgaatgtggt gttaaagctac tgagcgcttc tctcgtctcc gttctccgct ctgggtgcat gtcccatac) and oBN478 (gtacatggat gatatagata tg) for amplification of PrM4 in the deletion site 4; oBN225 (ccttaatcga attctcatgt catggatggg gtaaccagca ttaatagt) and oBN479 (gctcccattc aattcacatt gg) for amplification of PrM3 in IGR I4L/I5L; oBN194 (atgttgaaca taatgaacag gaggaaaaga tctgtgacca tgctcctcat gctgctgccc acagccctgg cgttccatct) and oBN476 (gattttgcta ttcagtggac tggatg) for amplification of PrM1 in the deletion site 2. DNA was extracted as described in the specification. For sequencing the Expand Polymerase (Roche, Germany, Mannheim), the Quick Start Kit (Beckman, Germany, Unterschleißheim) and oligonucleotides from MWG, Germany, Ebersberg were used.

12. Transfection: CEF were seeded in VP-SFM (+ 4mmol/l L-Glutamine) in 6-well plates. At day 1, the stock of the MVA, in which Denguevirus PrM genes were to be inserted, was diluted in VP-SFM/L-Glutamine so that 500-µl dilutions contained an appropriate amount of virus that gave a moi (multiplicity of infection) of 1. Medium was removed

from cells and the cells were infected for 1 hour with diluted virus at room temperature. The virus inoculum was removed and cells were washed with DMEM/VP-SFM. 1-2 µg of linearized recombination vector per transfection were mixed with buffer EC (Effectene Transfection Kit, Qiagen, Germany, Hilden) to give a final volume of 100 µl. 3.2 µl Enhancer were added and the mixture was vortexed and incubated at room temperature for 5 min. 10 µl of Effectene were added and after mixing thoroughly by vortexing the transfection set up was incubated at room temperature for 10 min. 600 µl of VP-SFM/L-Glutamine were added and the resulting transfection mix was added to the cells, which were already covered with medium (1.6 ml). The transfection was incubated at 37°C with 5%CO_2 over night. At day 2, the medium was removed and replaced with fresh VP-SFM/L-Glutamine and the incubation at 37°C with 5%CO_2 over night was continued. At day 3, the cells were scraped into the medium, the cell suspension was transferred to an adequate tube and frozen at –20°C for short-term storage or at –80°C for long-term storage.

13. Isolation and screening of new recombinant MVA constructs: CEF cells were seeded in VP-SFM (+ 4mmol/l L-Glutamine) in 6-well culture plates and cultivated to 90-100% confluence. Cell extracts from the infected/transfected cells above were diluted serially in VP-SFM/L-Glutamine so that the dilution contained an appropriate amount of virus that gave single foci (10^{-2}-10^{-6}). Medium was removed from cells and infected for 1 hour rocking at room temperature with 500 µl of the virus dilution for a 6-well. Virus inoculum was removed from 6-wells and cells were washed with VP-SFM. 2 ml low melting agarose were mixed with the appropriate selection medium and infected cells were covered with an agarose overlay. Incubation was performed at 37°C with 5%CO_2 for 48 hours. Single foci under the agarose overlay were marked and isolated by picking them with a glass pipette or a pipette tip. The obtained agarose slice was re-suspended in 200 µl appropriate medium in a 1.5 ml vial. The isolated virus was released from the agarose slice by three cycles of freeze-thawing, ultrasonication or vigorous vortexing alternatively. 100 µl of the virus suspension were then transferred on test cells (BHK or CEF) in 12-well plates, which show 80-100% confluence, and incubated for 48 hours to allow the amplification of the recombinant virus. After 48 h, the medium was removed

and 300 µl PBS per well were added. The cells were scraped in PBS. 200 µl of the virus suspension were used for DNA preparation for screening. The DNA was extracted using the Nucleospin Blood DNA kit according to the manufacturers protocol. A primer set specific for detection of the insertion site and for detection of MVA-BN empty vector was used. The PCR set-up was performed as follows in a total volume of 50 µl: DNA: 1.0 - 5.0 µl; H_2O : x µl; 10 x buffer + $MgCl_2$: 2.0 µl; dNTPs (10µmol):0.4 µl; Primer 1: 1.0 µl; Primer 2: 1.0 µl; Taq polymerase: 0.1 µl. 35 cycles under conditions suitable for the Primer set (annealing temperature) and the length of the expected PCR fragment (1 min extension per kb) were performed. Non-recombinant MVA-BN DNA was used as control for empty vector; the plasmid used for homologous recombination was used as positive control for insertion and additionally a water negative control was set up. The correct insertion of the foreign genes in the MVA genome was verified by sequencing. The sequenced region covered partly the flanking region, the promoter necessary for the expression of the foreign gene and the coding region of the inserted gene.

14. Isolation of MVA-mBN12 (recMVA-PrM-1+2+3+4): For the generation of a recombinant MVA-BN comprising PrM genes from Denguevirus serotype 1, 2, 3 and 4, genes from Dengue serotypes 1 and 4 were inserted into one MVA-BN and genes from Denguevirus 2 and 3 were inserted into a second MVA-BN. The two different recombinant MVA-BN were mixed and used to infect cells. It was checked whether recombinants could be obtained that comprise PrM genes from Denguevirus serotypes 1, 2, 3 and 4. More specifically the experimental setting was as follows: pBN40 (comprising the Denguevirus 4 PrM gene flanked by MVA sequences from deletion site 4) was transfected in MVA infected CEF cells. Recombinant virus was purified by 4 rounds of plaque purification under selective conditions. The purified recombinant virus was used to infect CEF cells that subsequently were transfected with pBN39 (comprising the Denguevirus 2 PrM gene flanked by MVA sequences from deletion site 1). The double recombinant virus was purified by 16 rounds of plaque purification, resulting in MVA1. In a separate assay pBN50 (comprising the Denguevirus 3 PrM gene flanked by sequences of the I4L/I5L intergenic region) was transfected in MVA infected CEF-cells. The recombinant virus was purified by 3 rounds of plaque purification under selective

conditions. The resulting recombinant virus was termed MVA2. Subsequently, pBN49 (comprising the Denguevirus 1 PrM gene flanked by MVA sequences from deletion site 2) was transfected in MVA2 infected CEF-cells. The recombinant virus was purified by 10 rounds of plaque purification under selective conditions. The resulting virus was termed MVA 3. To obtain the virus with up to four inserts MVA 1 and MVA 3 were used for co-infection of CEF cells. Purified virus was obtained by 10 rounds of amplification under selective conditions. 12 plaques of the resulting virus mBN12 were isolated.

15. One goal of the experiments described herein was the creation of a recombinant MVA, which contains the PrM coding sequence of up to four serotypes of Denguevirus (1, 2, 3 and 4). The sequences inserted in the MVA are highly homologous sequences encoding antigens of different serotypes of denguevirus. Therefore the stability of the resulting recombinant MVAs was analysed. Additionally different insertion sites were used in each approach in order to proof the general validity of the results.

16. 5.1 Analysis of MVA-mBN12 (recMVA-PrM-1+2+3+4): Four recombination vectors were used for the insertion of the PrMs of four serotypes of Denguevirus in the MVA genome: pBN49 for the insertion of PrM1 in Deletion 2; pBN39 for the insertion of PrM2 in Deletion 1; pBN50 for the insertion of PrM3 in the intergenic region IGR I4L-I5L and pBN40 for the insertion of PrM4 in Deletion 4. The insertion sites in the MVA genome are shown in Figure 2 attached hereto as Appendix C. Using the technique described above, a mixture of clones with one, two, three and even four insertions was expected. Twelve (12) clones of the resulting recombinant MVAs were analyzed by PCR with primers specific for each insertion (See Figure 3, attached hereto as Appendix D). As it can be seen, MVA clones with inserts of the PrM encoding sequence of two serotypes (1, 3, 9, 11), three serotypes (6,10,12) and even four serotypes (2) were obtained. These clones were stable over multiple rounds of amplification. Therefore recMVAs containing two, three and even four homologous sequences are stable.

17. Discussion: Four serotypes of Denguevirus are described, wherein the sequence differ only in a small number of nucleotide positions and have exactly the same length. For

example the PrM protein of one serotype is between 71 and 77 % homologous to the PrM encoding sequence of the other serotype and has the same length. According to the data shown in this Declaration, at least three homologous sequences can be inserted into a poxviral vector. DNA of each mBN12 clone was used as template for PCR amplification, wherein primer pairs specifically amplifying the sequence encoding the PrM protein of one serotype were used. As can be seen in Figure 3 (Appendix C), MVA clones with inserts of the PrM encoding sequence of two serotypes, three and even four serotypes were obtained. It is my opinion that this data demonstrates that these clones were stable over multiple rounds of amplification, i.e. clones with two, three or four inserts did not loose an insert due to homologous recombination between the PrM coding sequences.

18. It is my opinion that the results shown herein demonstrate that an MVA recombinant comprising several highly homologous dengue viral sequences is stable over multiple rounds of amplification and does not undergo homologous recombination. The experiments resulted in a MVA vector, which has inserts of the sequence encoding PrM protein of different dengue virus serotypes. Evidence for the insertion of the PrM encoding sequence of several serotypes is provided by serotype specific PCR.

19. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 17.03.08

S. Leyrer

SONJA LEYRER